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ARTICLE

Simultaneous Degradation of Mixed Insecticides by Mixed Fungal Culture Isolated from Sewage Sludge

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The degradation of mixed (DDT and chlorpyrifos) insecticides by mixed insecticide enriched cultures was investigated. The mixed fungal population was isolated from mixed insecticide acclimatized sewage sludge over a period of 90 days. Gas chromatography was used to detect the concentration of mixed insecticides and calculate the degradation efficiency. The results showed that the degradation capability of the mixed microbial culture was higher in low concentrations than in high concentrations of the mixed insecticides. After 12 weeks of incubation, mixed pesticide enriched cultures were able to degrade 79.5−94.4% of DDT and 73.6−85.9% of chlorpyrifos in facultative cometabolic conditions. The fungal strains isolated from the mixed microbial consortium were identified as *Fusarium* sp. isolates GFSM-4 (ITCC 6841) and GFSM-5 (ITCC 6842). The fungal culture GFSM-4 could not utilize mixed insecticides as source of carbon and nitrogen, probably due to high combined toxicity of the mixed insecticides. Liquid media deficient in carbon (1% mannitol) and nitrogen (0.1% sodium nitrate) source increased the degradation efficiency of DDT and chlorpyrifos to 69 and 45%, respectively. The media with normal carbon and deficient nitrogen (0.1% sodium nitrate) sources extensively increased the degradation efficiencies of DDT (94%) and chlorpyrifos (69.2%). Traces of *p,p′*-dichlorobenzophenone and desdiethylchlorpyrifos were observed in the liquid medium, which did not accumulate probably due to further rapid degradation. This fungal isolate (GFSM-4) was able to degrade simultaneously DDT (26.94%) and chlorpyrifos (24.94%) in sterile contaminated (50 mg of each insecticide kg^{-1}) soil in aerobic conditions.

KEYWORDS: Mixed pesticides; DDT; chlorpyrifos; insecticide mixture; biodegradation; fungal degradation; sewage sludge; simultaneous degradation; contaminated soil

INTRODUCTION

DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane] is one of the most important contemporary insecticides. Although banned for use in agriculture for a few decades now due to serious environmental problems, DDT has been spectacularly successful in public health vector control, particularly in the control of malaria (*1*) in India and other developing countries. DDT has a reported half-life in the environment of 2−15 years in most soils and has been classified as one of the most persistent organic pollutants (POPs). Chlorpyrifos [*O*,*O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate], a chlorinated organophosphorus insecticide, is effectively applied in a wide range of field crops against several insect pests and as preconstruction treatment for termite control in high doses (*2*). This chlorinated organophosphorus insecticide is known to persist in soil for 3−12 months and even longer depending upon the dose and is one of the most persistent organophosphorus (OP) insecticides.

There are several reports of a wide range of water and terrestrial ecosystems becoming contaminated with these pesticides. Due to environmental concerns associated with the accumulation of these pesticides in food products and water supplies, efforts are currently underway to develop safe, convenient, and economically feasible methods for pesticides detoxification. Current methods to detoxify pesticides mainly rely on chemical treatment, incineration, and landfills (*3*). Chemical methods, although feasible, are problematic due to the production of a large volume of acids and alkali that subsequently must be disposed of. Landfills function adequately, but leaching of pesticides into the surrounding soil and groundwater supplies is a big concern. Incineration, which is the most reliable method for destruction of these compounds, has met serious public opposition because of the potentially toxic emissions (*4*). Soil microflora is another potential candidate for detoxification of pesticides. There are reports that soil contaminated with pesticides could be decontaminated by inoculation with specifically adapted microorganisms (*5*). Only a few studies have been reported so far on the degradation of mixtures of pesticides (*6*) and none on DDT−chlorpyrifos mixture.

Under aerobic conditions, these pesticides are dechlorinated into metabolites more susceptible to further degradation (*7*). A chlorpyrifos-degrading bacterium, *Klebsiella* sp., isolated from an activated sludge of a wastewater treatment plant, was able to degrade 92% of chlorpyrifos as sole carbon source (*8*). A *Sphingomonas* sp. isolated from a polluted treatment system of a chlorpyrifos manufacturer utilized chlorpyrifos as its sole

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source of carbon for growth, by hydrolyzing chlorpyrifos to 3,5, 6-trichloro-2-pyridinol (*9*). Degradation of DDT by the white rot fungus *Phanerochaete chrysosporium* was demonstrated to form the metabolites DDD, dicofol, and *p*,*p*′-dichlorobenzophenone (DBP) (*10*). There has been no report, so far, on the biodegradation of these two mixed insecticides.

The objective of the present study was to isolate a mixed fungal culture from a DDT−chlorpyrifos mixture enriched sewage sludge sample and to assess the ability of isolated cultures to degrade simultaneously these two insecticides present as mixed contaminant in culture media and soil.

MATERIALS AND METHODS

Pesticides and Chemicals. Analytical grade chlorpyrifos (97.0% purity) was supplied by M/s Rallis India Ltd. Trichloropyridinol (TCP), a degradation product of chlorpyrifos, was prepared in the laboratory (*11*). Analytical grade *p*,*p*′-DDT (98.0% purity) and its two degradation products, *p*,*p*′-DDE and DBP, were procured from M/s Hindustan Insecticides Ltd. (HIL). HPLC and analytical grade solvents and chemicals were purchased from E. Merck India Ltd. Analytical grade reagents used in medium preparation were procured from E. Merck India Ltd. The culture media used in fungal isolation and characterizations were autoclaved at a pressure of 15 psi at 110 °C for 20 min.

Sewage Sludge. The sewage sludge was procured from Sewage Disposal Plant sight in New Delhi, India. The dried sludge was sieved through a 2 mm sieve for use.

Soil. Soil used in the study was collected from an agricultural field (up to 15 cm depth) of the Indian Agricultural Research Institute (IARI), New Delhi, India. The soil was characterized as sandy loam (64% sand, 19% silt, and 17% clay) with a pH of 8.1 and 0.5% organic matter. Prior to use, soil was sieved through a 2 mm sieve.

Isolation and Characterization of Fugal Cultures from Sewage Sludge. The sample (50 g) of solid sewage sludge in a glass bottle (250 mL) was treated with a DDT−chlorpyrifos (5 mg each) mixture in 1 mL of acetone solution, thoroughly mixed, and left undisturbed for 90 days for acclimatization.

A sample (1 g) of acclimated sewage sludge was used to inoculate an Erlenmeyer flask containing 20 mL of Czapek Dox liquid medium amended with the mixed insecticides for aerobic fungus cultivation (*12*). The Czapek Dox liquid medium consisted of (g L^{-1}) $NaNO_3$, 3.0; K_2HPO_4, 1.0; $MgSO_4 \cdot 0.7H_2O$, 0.5; $FeSO_4 \cdot 0.7H_2O$, 0.01; and sucrose 30.0, dissolved in 1 L of distilled water. The flasks were incubated at 28 ± 1 °C in Biological Oxidation Demand (BOD) with intermittent shaking. After 72 h, it was then used as inoculum for transferring to flasks containing medium and incubated under similar conditions. One milliliter of enriched medium was transferred into freshly prepared enrichment media every 3 weeks. At each culturing step, the concentration of mixed (DDT−chlorpyrifos) insecticides was increased from 10 to 20, 50, and 100 mg of each of the two insecticides per liter of culture medium.

A loopful inoculum of final enriched medium was spread-plated on a Czapek Dox agar plate containing DDT−chlorpyrifos (50 mg each L^{-1}) mixture in triplicate and incubated at 28 ± 2 °C for 3 days. Representative mixed fungal culture from sewage sample was selected for use. Further purification resulted in isolation of pure cultures. The single colonies were streaked on potato dextrose agar plates and incubated at 28 ± 2 °C for 2−3 days. Streaking of colonies with the same morphology (*13*) was carried out three times, and the pure isolates were stored at 4 °C until further use.

The isolates that were capable of degrading both DDT and chlorpyrifos were sent to the Indian Type Culture Collection (ITCC), Division of Plant Pathology, IARI, New Delhi, India, for identification.

Degradation of Mixed Insecticides by Isolated Mixed Fungal Culture in Liquid Medium. For degradation study, 10 mL of sterilized nutrient medium in an Erlenmeyer flask amended with mixed (10 mg each L^{-1}) insecticides in 0.1 mL of acetone solution was inoculated with a 0.2 mL suspension of mixed fungal culture (1.22×10^{12} cfu mL^{-1}). Control comprised 10 mL of sterilized nutrient solution with mixed (10 mg each L^{-1}) insecticides but not inoculated with the fungal culture. The treatments were carried out in triplicate.

In another experiment to evaluate the influence of deficient nutrients (C and N), sterilized Czapek Dox liquid medium (10 mL) containing mixed (50 mg each L^{-1}) insecticides without and with partial (1%) carbon and/or partial (0.1%) nitrogen source in triplicate was also inoculated with a 0.2 mL suspension of isolated mixed culture along with respective controls and incubated under identical conditions simultaneously. After 3 weeks, treatments and their controls in triplicate were taken for insecticide analysis. The percentage of DDT and chlorpyrifos degradation was calculated on the basis of the amount present in each treatment compared with the initial amount of DDT and chlorpyrifos.

To evaluate the adsorption of the insecticides to the cellular membranes, mixed culture previously autoclaved at 110 °C for 20 min was used as inoculum in control samples.

Degradation of Mixed Insecticides by Enriched Fungal Culture in Sterile Soil. Sandy loam soil samples (10 g) contained in Erlenmeyer flasks were sterilized in an autoclave at a pressure of 15 psi at 110 °C for 20 min. Half of the flasks containing sterilized soil were aseptically treated with 0.5 mL of acetone solution of mixed insecticides (50 mg each kg^{-1}) and inoculated with a 0.2 mL suspension of isolated mixed culture. The remaining control flasks containing sterile soil samples were aseptically treated with 0.5 mL of acetone solution of mixed insecticides (50 mg each kg^{-1}) but not inoculated with fungal cultures. Samples were incubated in BOD for 2 weeks at 28 ± 2 °C with intermittent shaking. Triplicate inoculum treated and control flasks were removed at 0, 7, and 15 days for analysis of DDT and chlorpyrifos contents in soil samples.

Analysis of DDT, Chlorpyrifos, and Metabolites. DDT and chlorpyrifos were extracted from Czapek Dox culture medium by liquid−liquid partitioning and from soil by shaking (*14*). The efficiency of the extraction process was measured by spiking known concentrations of DDT−chlorpyrifos mixture in the matrix.

Sample of culture medium (10 mL) was extracted with dichloromethane (20 mL) and shaken vigorously for 5 min in a standard separating funnel with a Teflon stopper. The water layer was decanted carefully and was extracted with 10 mL of dichloromethane twice more. Soil sample (10 g) was extracted with acetone (3 × 25 mL) by shaking on a horizontal shaker for 20 min. The solvent extracts were dehydrated by passing through anhydrous sodium sulfate, and solvent was evaporated on a rotary evaporator under vacuum to dryness. The residue was dissolved in a hexane:acetone mixture (4:1), the volume was made up, and the mixture was finally analyzed by gas chromatography with electron capture detection (*15*, *16*). The residue was dissolved in acetonitrile for analyzing insecticide derivatves by HPLC (*17*).

The gas chromatograph (Shimadzu model GC-17A) fitted with an electron capture detector (GC/ECD) and a megabore column (25 m × 0.53 μm i.d.; OV-1) was used. The GC operating conditions were as follows. The column was held initially at a temperature of 190 °C for 4 min, then increased at 10 °C min^{-1} to 230 °C, and finally held at that temperature for 4 min, with a total run time of 13 min. The temperatures of the injector port and detector were maintained at 250 and 300 °C, respectively. Nitrogen was used as carrier gas at a flow rate of 30 mL min^{-1}. A 3 μL aliquot of the sample was injected and chromatogram recorded. Under these conditions the compounds eluted with distinct retention times (Rt) as *p*,*p*′-DBP (1.800 min), *p*,*p*′-DDE (2.766 min), *o*,*p*′-DDT (4.212 min), chlorpyrifos (4.779 min), and *p*,*p*′-DDT (4.978 min) and were identified by cochromatography with authentic products on GC.

A Hewlett-Packard HPLC instrument (series 1100) equipped with a degasser, quaternary pump, photodiode array detector (at λ_{max} 254 nm) connected with a loop injection system, and a computer (Model Vectra) was used. The stationary phase consisted of a Lichrosphere RP-18 packed stainless steel column (250 mm × 4 mm i.d.). The mobile phase was acetonitrile:acidic water (1% H_3PO_4, 70:30) maintained at a flow rate of 1.0 mL min^{-1}. A 20 μL aliquot of the sample volume was injected each time, and chromatograms were recorded in a Windows 95 NT based HP Chemstation program. Under these conditions, the compounds eluted at Rts of 2.318 min (desdiethyl chlorpyrifos), 2.576 min (tricholropyridinol, TCP), 4.063 min (*p*,*p*′-DBP), 4.251 min (chlorpyrifos), 5.884 min (*p*, *p*′-DDT), and 6.853 min (*p*,*p*′-DDE).

The selected samples were also analyzed by GC-MS-MS and LC-MS-MS (*18*) for identification of DDT and chlorpyrifos metabolites. A Shimadzu instrument fitted with a mass selective detector using a capillary column (DB-5, 30 m × 0.2 mm i.d.) operated with splitless injection was

Table 1. Recovery of DDT and Chlorpyrifos from Liquid Media Treated with a Mixture of Equal Concentrations of Insecticides and Degradation by Enriched Mixed Fungal (*Fusarium* sp.) Cultures Obtained from Enriched Sewage Sludge in 20 Days

	DDT		chlorpyrifos	
insecticide concn (mg L^{-1})	amount recovered[a] (mg L^{-1})	degradation (%)	amount recovered[a] (mg L^{-1})	degradation (%)
10	0.55 ± 0.065	94.45 ± 0.65	1.11 ± 0.09	85.90 ± 0.9
20	1.97 ± 0.41	90.15 ± 2.05	3.63 ± 0.18	81.85 ± 0.9
50	10.24 ± 0.6	79.54 ± 1.21	13.17 ± 0.56	73.65 ± 1.13
100	77.46 ± 1.2	22.54 ± 1.2	79.12 ± 0.8	20.88 ± 0.8

[a] Values reported are mean ± standard deviation of three replicates.

used. Operating temperatures were injection port, 250 °C, column programmed from 100 to 250 °C at a rate of 15 °C min^{-1}. Helium was used as carrier gas (2 mL min^{-1} flow). The mass spectrometer was operated at an ionization potential of 70 eV under electron potential. LC-MS-MS was carried out using a Thermo Electron MS spectrometer. Detection of mass was done by an electron spray ionization (ESI) source with a Finnigan LCQ tune plus program fitted with a MAX detector. Xcalibur software was used for the purpose of identification and fragmentation of required masses. The following MS parameters were optimized in direct infusion mode: spray voltage, 3.5−5 kV; sheath gas flow rate, 25 mL min^{-1}; auxiliary gas flow rate, 5 mL min^{-1}; spray current, 0.5 μA; capillary temperature, 240 °C; capillary voltage, 20−35 V; and tube lens offset, 40−65.

RESULTS AND DISCUSSION

The sensitivity of the GC method for the two insecticides was 0.01 mg L^{-1} solution, and the instrument detection limit was 30 pg each for DDT and chlorpyrifos. Mean extraction recoveries from Czapek Dox liquid medium and soil were 94.9 and 79.7%, respectively, for DDT and 87.5 and 81.5%, respectively, for chlorpyrifos.

Degradation Studies with Mixed Culture in Liquid Medium. The decrease in concentration of the two insecticides in Czapek Dox liquid medium inoculated with mixed fungal culture compared to that not inoculated was considered as fungal degradation. The GC analysis confirmed substantial removal of the two insecticides (**Table 1**). Mean degradation of DDT was 94.45, 90.15, and 79.54%, respectively, and that of chlorpyrifos was 85.9, 81.8, and 73.6%, respectively, at 10, 20, and 50 mg each L^{-1} initial concentration levels. The degradation of both the insecticides was highest at lowest concentration and declined with increasing levels. At the highest concentration (100 mg each L^{-1}) degradation was very low (∼20%). This was probably due to the poor fungal growth observed in the media. Several workers have reported that microbial growth and concomitant biodegradation of insecticides are hindered by the toxicity exerted by high concentrations of the substrate itself (*19*, *20*).

Degradation of both DDT (80−94%) and chlorpyrifos (74−86%) simultaneously in the aerobic system within the first 20 days showed that the enriched mixed culture was able to degrade/mineralize the DDT−chlorpyrifos mixture cometabolically. Krishna and Philip also observed facultative cometabolic degradation of mixed (lindane, methyl parathion, and carbofuran) pesticides by mixed pesticide enriched culture after 7 weeks of incubation (*6*).

Fungal Culture Identification. High degradation capability shown by the mixed fungal culture isolated from adapted sewage sludge for a mixture of the two insecticides enriched with 50 mg each L^{-1} concentration was selected for identification. The mixed microbial population on purification was separated into two culture isolates designated GFSM-4 (ITCC 6841) and GFSM-5 (ITCC 6842). These were identified on the basis of cultural and morphological details as *Fusarium solani*, *Fusarium oxysporum*, and *Fusarium moniliforme*. Of the two isolates, GFSM-4 showed a higher ability to degrade DDT and chlorpyrifos in liquid medium (results not shown) and, hence, was selected for further studies.

Table 2. Influence of Nutrients (Mannitol and Sodium Nitrate) on Simultaneous Degradation of DDT and Chlorpyrifos by Enriched *Fusarium* sp. Isolate GFSM-4 Obtained from Enriched Sewage Sludge

	control		treatment	
nutrient	amount recovered[a] (mg L^{-1})	degradation (%)	amount recovered[a] (mg L^{-1})	degradation (%)
	DDT			
normal C and N	49.30 ± 0.75	0.2 ± 0.02	40.70 ± 2.7	18.60 ± 0.15
1% C	49.35 ± 0.8	0.2 ± 0.01	49.25 ± 1.25	1.50 ± 0.05
0.1% N	49.25 ± 0.7	0.4 ± 0.01	2.98 ± 0.89	**94.03 ± 5.1**
1% C and 0.1% N	49.05 ± 0.75	0.5 ± 0.02	17.62 ± 2.55	64.78 ± 2.31
	Chlorpyrifos			
normal C and N	44.70 ± 1.38	1.10 ± 0.0	34.55 ± 1.05	30.9 ± 2.1
1% C	44.85 ± 1.56	1.02 ± 0.0.01	46.52 ± 3.1	6.96 ± 6.24
0.1% N	44.53 ± 1.21	1.20 ± 0.01	15.4 ± 2.12	**69.2 ± 4.24**
1% C and 0.1% N	43.96 ± 1.82	1.30 ± 0.005	25.17 ± 2.1	49.66 ± 3.78

[a] Values reported are mean ± standard deviation of three replicates.

Influence of Carbon and Nitrogen on Degradation of DDT−Chlorpyrifos Mixture. The mixed (DDT and chlorpyrifos) insecticides (50 mg of each insecticide L^{-1}) enriched fungal isolate GFSM-4 was used in subsequent degradation studies. Experiments were carried out in Czapek Dox medium without and with carbon and/or nitrogen source. Abiotic degradation of the insecticides was also evaluated by amending liquid medium with an equal amount of the insecticide mixture but without fungal inoculum.

There was no degradation observed of the two insecticides by fungal isolate GFSM-4 in liquid medium deficient in normal carbon (sucrose) and nitrogen (sodium nitrate) sources due to nontolerance and extremely poor growth of the isolate in the culture medium, indicating that the mixed insecticides could not be utilized as sole carbon and/or nitrogen sources by the fungal isolate.

There were reports that the addition of glucose/sucrose did not increase the degradation efficiency of pesticide (*21*). Hence, mannitol was used as an auxiliary carbon source in the present study. The degradation studies were repeated with mannitol (carbon) and sodium nitrate (nitrogen) to check the influence of auxiliary carbon and nitrogen on DDT−chlorpyrifos degradation. The enriched fungal isolate GFSM-4 showed marginal cometabolic degradation ability (18.6% DDT and 30.9% chlorpyrifos) in the medium with normal carbon and nitrogen sources. In the presence of 1% carbon source and normal nitrogen source, negligible DDT (2%) and chlorpyrifos (6%) degradation indicated that fungal culture could not utilize the DDT−chlorpyrifos mixture as carbon source (**Table 2**). Earlier Awasthi et al. also reported no increase in degradation efficiency due to the addition of secondary carbon (*21*). Liquid medium with partial mannitol (1%) and partial sodium nitrate (0.1%) enhanced the degradation efficiency of DDT (64.8%) and chlorpyrifos (49.7%). However, in culture medium deficient only in sodium nitrate (0.1%), the degradation efficiency of DDT enhanced drastically to 94% and that of chlorpyrifos increased to 69.2%. The mixed fungal consortium in the present study preferred nutrient nitrogen-deficient

Table 3. Recovery of DDT and Chlorpyrifos from Sterile Soil Treated with a Mixture of Equal Concentrations (50 mg kg^{-1}) of Insecticides and Degradation by *Fusarium* sp. Isolate GFSM-4 in 20 Days

	control		treatment		
time (days)	amount recovered[a] (mg kg^{-1})	degradation (%)	amount recovered[a] (mg kg^{-1})	degradation (%)	fungal degradation (%)
			DDT		
0	41.64 ± 0.43	0	40.23 ± 0.56	0	
7	41.50 ± 0.83	0.34 ± 0.09	34.87 ± 0.67	15.01 ± 0.09	14.67 ± 0.07
15	41.50 ± 0.37	0.34 ± 0.1	29.45 ± 0.11	27.28 ± 0.38	26.94 ± 0.12
			Chlorpyrifos		
0	36.83 ± 0.99	0	35.21 ± 0.20	0	
7	15.36 ± 0.1	58.38 ± 0.16	13.97 ± 0.11	60.32 ± 0.5	1.94 ± 0.70
15	15.32 ± 0.07	58.28 ± 0.23	5.90 ± 0.105	83.22 ± 0.0.39	24.94 ± 0.63

[a] Values reported are mean ± standard deviation of three replicates.

cultures for mineralization of the mixed chlorinated insecticides. Earlier extensive biodegradation of DDT by the white rot fungus *P. chrysosporium* was also demonstrated by the disappearance and mineralization of [^{14}C]DDT in nutrient nitrogen-deficient cultures (*10*).

Previous researchers reported that the removal of DDT or chlorpyrifos took place simultaneously with the formation of intermediate metabolites such as DDE, dicofol, and DBP (*22*, *23*) or TCP and desdiethylchlorpyrios (*5*, *24*). In the present study, traces of DBP and desdiethylchlorpyrifos were observed in the liquid medium, which did not accumulate, probably due to further rapid degradation.

Isolation of relevant enzymes from the mixed fungal culture and comparison with those of other DDT- and chlorpyrifos-degrading microbes may give insight into the pathway/mechanism of these two insecticide mixture degradation.

Simultaneous Degradation of DDT−Chlorpyrifos Mixture by Fungal Isolate GFSM-4 in Contaminated Soil. The isolated fungal strain GFSM-4 was tested for its ability to grow under sterile conditions and to degrade mixed (DDT−chlorpyrifos) insecticides in contaminated soil.

Dissipation of DDT was negligible (0.34%) up to day 15 in sterile control soil without inoculum. Degradation of DDT by GFSM-4 in sterile soil was 15.01 and 27.28% in 7 and 15 days, respectively. The initial residues of 36.83 mg kg^{-1} chlorpyrifos dissipated to 15.36 and 15.32 mg kg^{-1} on days 7 and 15, respectively, in sterile control soil could be due to hydrolysis (**Table 3**). Degradation of chlorpyrifos by *Fusarium* sp. in sterile soil was 1.94 and 24.94% in 7 and 15 days, respectively. The fungal degradation of DDT was relatively faster (14.67%) compared to that of chlorpyrifos initially, that is, after 7 days of incubation. However, in next 7 days *Fusarium* sp. isolate GFSM-4 was able to degrade 26.94% of DDT and 24.94% of chlorpyrifos simultaneously (total 51.88%) in sterile soil.

The mixed pesticide-enriched culture GFSM-4 was capable of degrading DDT−chlorpyrifos mixtures in sterile soil without any toxic intermediate metabolites. Possibly, metabolites formed were rapidly degraded. Further studies under field conditions are, however, required for confirmation and recommending the usefulness of *Fusarium* sp. isolate GFSM-4 for bioremediation of soil contaminated with mixture of DDT−chlorpyrifos.

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